The HIV Env variant N283 enhances macrophage tropism and is associated with brain infection and dementia

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HIV infects tissue macrophages and brain microglia, which express lower levels of CD4 and CCR5 than CD4$^+$ T cells in peripheral blood. Mechanisms that enhance HIV tropism for macrophages in the CNS and other tissues are not well understood. Here, we identify an HIV envelope glycoprotein (Env) variant in the CD4-binding site of gp120, Asn 283 (N283), that is present at a high frequency in brain tissues from AIDS patients with HIV-associated dementia (HAD). N283 increases gp120 affinity for CD4 by decreasing the gp120-CD4 dissociation rate, enhancing the capacity of HIV Envs to use low levels of CD4 for virus entry and increasing viral replication in macrophages and microglia. Structural modeling suggests that the enhanced ability of Envs with N283 to use low levels of CD4 is due to a hydrogen bond formed with Gln 40 of CD4. N283 is significantly more frequent in brain-derived Envs from HAD patients (41%; $n = 330$) compared with non-HAD patients (8%; $n = 151$; $P < 0.001$). These findings suggest that the macrophage-tropic HIV Env variant N283 is associated with brain infection and dementia *in vivo*, representing an example of a HIV variant associated with a specific AIDS-related complication.

CD4 | envelope | neurotropism | microglia

HIV type 1 (HIV) infects macrophages and microglia in the CNS and causes HIV-associated dementia (HAD) or mild neurocognitive impairment in 10–20% of patients with AIDS (1). Most antiretroviral therapies have poor CNS penetration, so the brain is a reservoir for viral persistence. HIV variants in brain are genetically distinct from those in lymphoid tissues and other organs, and specific sequences in the viral envelope glycoprotein (Env) have been associated with brain compartmentalization (2–9). Furthermore, brain-derived Envs from HAD and non-HAD AIDS patients are genetically and biologically distinct (7, 8, 10–12). Rhesus macaque models of simian immunodeficiency virus (SIV) infection provide additional evidence that only a subset of strains are neurotropic (13–15). The capacity of HIV or SIV strains to replicate efficiently in macrophages has been correlated with increased neurotropism (9, 14, 16–19) and may also be linked to progression of HIV/SIV disease (15, 20, 21). However, mechanisms that enhance HIV replication in macrophages in the CNS and other macrophage-rich tissues such as lung, colon, and bone marrow are not well understood.

HIV Env, which is organized into trimers on virions, consists of the gp120 surface and gp41 transmembrane subunits. HIV entry into cells is initiated by a high-affinity interaction between gp120 and CD4, which induces a conformational change in gp120 that exposes the coreceptor-binding site (22). The interaction of CD4-bound gp120 with the coreceptor triggers a conformational change in gp120, which leads to a structural rearrangement in gp41 that enables fusion and virus entry. CCR5, the primary coreceptor used for infection of macrophages and microglia (17, 19, 23, 24), is the coreceptor used by most viruses isolated from brain (11, 17, 18, 23, 24).

Tissue macrophages and brain microglia express lower cell surface levels of CD4 and CCR5 than CD4$^+$ T cells in peripheral blood (25, 26). Requirements for CD4 and CCR5 are interdependent, with the requirement for each receptor being increased when the other component is present at limiting levels (22). HIV Envs with enhanced tropism for macrophages/microglia also have an increased capacity to mediate fusion with cells expressing low levels of CD4 and CCR5, suggesting that reduced dependence on CD4/CCR5 levels may enhance viral replication preferentially in the CNS (16, 20, 27, 28). However, mechanisms by which HIV acquires an enhanced capacity to enter cells when receptor levels are low are unclear.

To investigate mechanisms by which HIV acquires enhanced tropism for macrophages and microglia in the CNS, we analyzed sequences of neurotropic HIV Envs and identified a variant in the CD4-binding site of HIV gp120, Asn 283 (N283), that is present at a high frequency in brain from AIDS patients with HAD. Here, we show that N283 increases gp120 affinity for CD4, enhancing the capacity of HIV Envs to use low levels of CD4 and increasing viral replication in macrophages and microglia. Furthermore, we demonstrate that N283 is associated with brain infection and HAD *in vivo*.

Results

N283 Is Associated with Brain-Derived Envs and Reduced CD4 Dependence. Recently, we cloned and characterized HIV Envs from AIDS patients with HAD and showed that Envs with reduced dependence on CD4 and CCR5 levels are more frequent in brain compared with lymphoid tissues (48), suggesting that viral adaptation for replication in the CNS may select for variants with an enhanced capacity to enter cells expressing low levels of these receptors. To investigate mechanisms by which neurotropic Envs acquire the ability to use low CD4, we cloned full-length Envs from primary brain viral isolates MACS2-br, UK1-br, and UK7-br from three HAD patients and tested their ability to use low CD4 levels in cell-to-cell fusion assays. ADA Env, cloned from a macrophage-tropic blood viral isolate, was used as a

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Abbreviations: HAD, HIV-associated dementia; SIV, simian immunodeficiency virus; PBMC, peripheral blood mononuclear cells; MDM, monocyte-derived macrophages.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ890515–DQ890517 and DQ976408–DQ976434).

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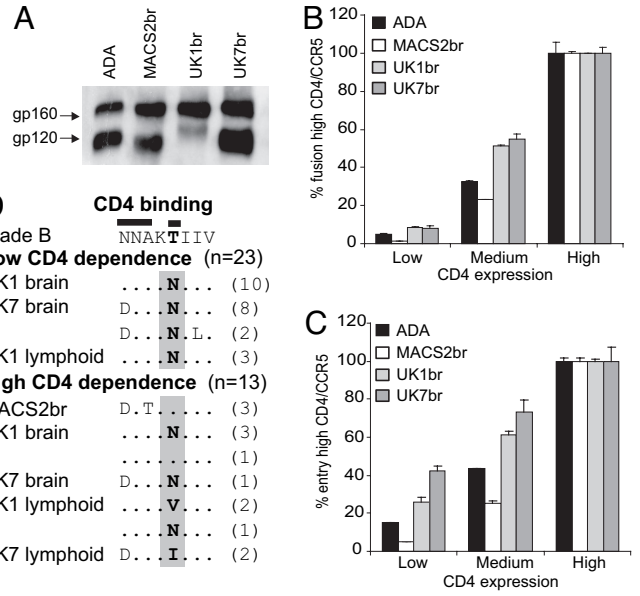

Fig. 1. N283 is associated with brain-derived Envs and low CD4 dependence. (*A*) 293T cells transfected with Env plasmids were analyzed by Western blotting with anti-gp120. (*B*) 293T cells expressing Env were analyzed in cell-cell fusion assays. (*C*) Cf2-Luc cells expressing different levels of CD4 and high CCR5 were infected with chimeric viruses expressing each Env, lysed 48 h postinfection, and analyzed for luciferase activity. Data in *B* and *C* were normalized to luciferase activity on cells expressing high CD4. Results shown are from duplicate samples and are representative of three Env clones from each viral isolate. Error bars represent standard deviations. (*D*) Amino acid sequences from the C2 region of gp120 were aligned by using Clustal X. Position 283 (numbered relative to the HXB2 reference sequence) is highlighted in gray. Env residues contacting CD4 in the HXB2 gp120 crystal structure (1G9M) (29) are indicated.

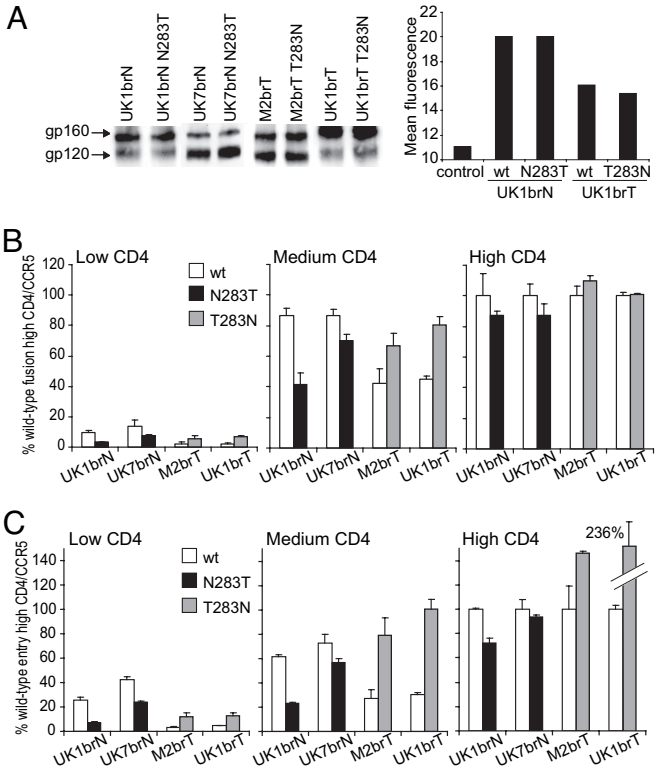

Fig. 2. N283 enhances the capacity of Envs to use low levels of CD4. (*A*) 293T cells were transfected with Env plasmids and analyzed by Western blotting with anti-gp120 (*Left*) or incubated with HIV+ patient sera and analyzed for Env cell surface expression by flow cytometry (*Right*). (*B*) 293T cells expressing Env were analyzed in cell-cell fusion assays. (*C*) Cf2-Luc cells expressing different levels of CD4 and high CCR5 were infected with recombinant viruses expressing each Env, lysed 48 h postinfection, and analyzed for luciferase activity. Data in *B* and *C* were normalized to luciferase activity of the wild-type Env on cells expressing high CD4. Results shown are from duplicate samples. Error bars represent standard deviations.

positive control. UK1-br and UK7-br Envs had an enhanced capacity to use low or intermediate levels of CD4 to mediate fusion and entry compared with ADA and MACS2-br Envs, which required higher levels of CD4 (ref. 28 and Fig. 1 *A–C*).

We analyzed 36 full-length Env amino acid sequences from patients MACS2, UK1, and UK7 [nine Envs cloned from viral isolates and 27 Envs cloned directly from brain and lymphoid tissues for variability in the 26 residues in gp120 that contact CD4 in the HXB2 gp120 crystal structure (1G9M; ref. 29). Most CD4 contact residues were conserved in the dataset. However, at position 283 in the C2 region of gp120, all 23 Envs with low CD4 dependence had Asn, whereas 8 of 13 Envs with high CD4 dependence had Thr (similar to the Clade B consensus), Val, or Ile (Fig. 1*D*). N283 was present in 24/28 brain Envs and four of eight lymphoid Envs. In contrast to most CD4 contact residues, position 283 is highly variable and under positive selection (30). These results suggest that the N283 variant is more frequent in brain than in lymphoid tissues from two patients and is also present at a high frequency in Envs with low CD4 dependence.

N283 Contributes to Reduced CD4 Dependence. To investigate whether N283 contributes to reduced CD4 dependence, we used mutagenesis to introduce N283T mutations in Envs with low CD4 dependence (UK1-br and UK7-br, hereafter referred to as UK1brN and UK7brN) and T283N mutations in Envs with high CD4 dependence (MACS2-br, hereafter referred to as M2brT, and UK1brT; ref. 28). The parental and mutant Envs were processed to gp120 and expressed on the cell surface at similar levels (Fig. 2*A* and data not shown). The N283T change in UK1brN and UK7brN Envs resulted in a 2.5- to 3-fold decrease in the ability to use low CD4 for fusion and entry, whereas the T283N change in M2brT and UK1brT Envs resulted in a 2.5- to 5-fold increase in the capacity to use low CD4 (Fig. 2 *B* and *C*). This increase was due to the presence of Asn at position 283, because a T283I change in UK1brT had no effect on CD4 dependence (Fig. 5, which is published as supporting information on the PNAS web site). Together, these data demonstrate that N283 contributes to reduced CD4 dependence in brain Envs from three patients.

N283 Enhances Macrophage and Microglia Tropism. To investigate the effect of N283 on macrophage and microglia tropism, we cloned wild-type and N283T mutant UK1brN Envs into pNL4–3 and infected peripheral blood mononuclear cells (PBMC), monocyte-derived macrophages (MDM), and microglia in primary human brain cultures. JC53 cells, which express high levels of CD4 and CCR5, were used as a positive control. UK1brN and N283T mutant viruses had similar replication kinetics in JC53 cells and PBMC (Fig. 3*A* and data not shown). In contrast, the virus containing the UK1brN N283T Env had delayed replication kinetics and reduced cytopathic effects in MDM and microglia compared with the virus containing the parental Env (Fig. 3*B*). Both viruses induced large syncytia in JC53 cells (data not shown). However, the parental virus induced large syncytia in MDM and microglia, whereas syncytia formation was reduced in cultures infected with the N283T virus (Fig. 3*B*). Similar results were seen with viruses containing wild-type and N283T mutant UK7brN Envs (data not shown). These data demonstrate

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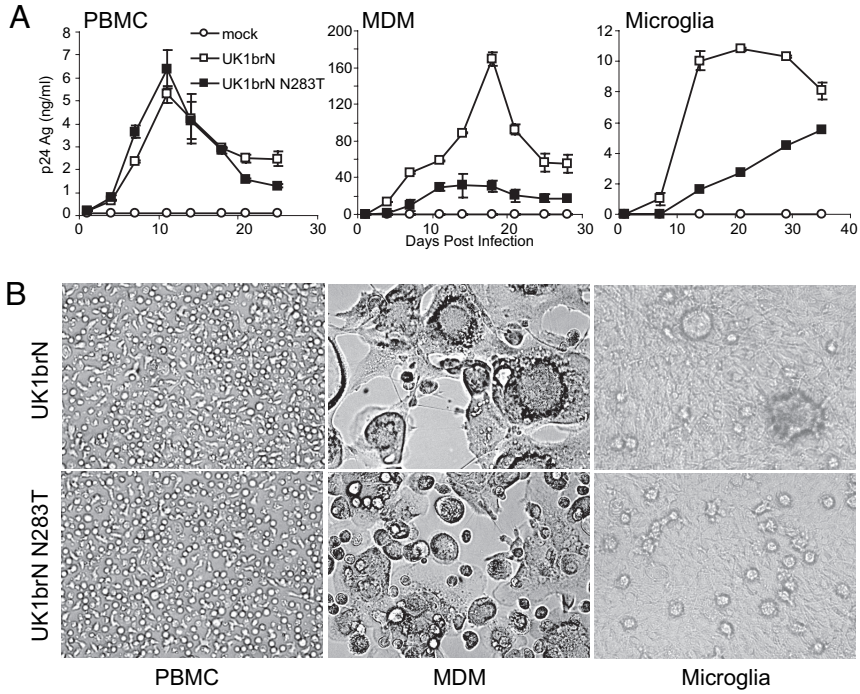

Fig. 3. N283 enhances macrophage and microglia tropism. PBMC, MDM, and microglia in primary brain cultures were infected as described in *Materials and Methods*. (*A*) Supernatants were collected every 3–7 days, and replication was monitored by p24 ELISA. Results shown are from triplicate samples. Error bars represent standard deviations. (*B*) Cytopathic effects in PBMC (day 11 postinfection), MDM (day 14 postinfection), and microglia (day 14 postinfection). Photographs were taken at a ×200 magnification.

that the N283 variant enhances macrophage and microglia tropism and also enhances cytopathic effects in cultured MDM and microglia.

N283 Increases CD4 Affinity by a Hydrogen Bond to CD4 Q40. To investigate the mechanism by which N283 contributes to low CD4 dependence, we modeled the T283N change in the JRFL gp120 crystal structure (2B4C; ref. 31). The HIV Clade B consensus amino acid at position 283 is Thr (Fig. 4*A Left*). Modeling with Swiss PDB Viewer (http://ca.expasy.org/spdbv) indicates an increased potential for N283 to form a hydrogen bond contact with CD4 Q40 (Fig. 4*A Right*). Similar results were obtained with the YU2 crystal structure (1G9N; ref. 29). Energy minimization of the theoretical structure confirmed these predictions (data not shown). These findings suggest that N283 may increase gp120-CD4 affinity, stabilize gp120-CD4 interactions, or affect the conformation of gp120 at the gp120-CD4 interface.

To determine whether the enhanced capacity of N283 to use low CD4 is due to an increased potential to form a hydrogen bond with CD4 Q40, we used mutagenesis to construct CD4 Q40A. Wild-type and mutant CD4 were expressed at equivalent levels on the cell surface (Fig. 4*B*). In entry assays, ADA and UK1brN viruses used low levels of wild-type CD4 more efficiently than UK1brN N283T and M2brT viruses (Fig. 4*C*). However, these viruses used CD4 Q40A with similar efficiencies (Fig. 4*C*), suggesting that the enhanced ability of Envs with N283 to use low CD4 is due to the capacity to form a hydrogen bond with Q40.

To investigate whether N283 influences the affinity of gp120 for CD4, we purified UK1brN wild-type and N283T mutant soluble gp120s (sgp120) from the supernatants of transfected 293T cells. sgp120 from YU2, a brain-derived envelope from a HAD patient, was used as a control, because its binding affinity for CD4 was determined previously (32). In contrast to ADA, YU2 does not have the N283 variant. Protein concentrations were verified by Coomassie staining of SDS/PAGE gels (Fig. 6, which is published as supporting information on the PNAS web site). CD4-binding affinities of 12.5–200 nM sgp120s were analyzed by BIACORE. The K_d of 6.42 nM obtained for YU2 is similar to reported values (4.3 nM; ref. 32; see also Table 1). UK1brN and N283T sgp120s had no significant difference in CD4 association rates. However, the dissociation rate of UK1brN N283T from CD4 was 3-fold higher than wild-type, resulting in a 2.5-fold decrease in binding affinity (Table 1 and Fig. 6). These results suggest that N283 increases affinity of the gp120 monomer for CD4 by decreasing the dissociation rate.

N283 Is Associated with Brain Infection and Dementia *in Vivo*. To determine whether the N283 amino acid variant is associated with brain infection *in vivo*, we examined 650 matched brain- and lymphoid-derived Envs from 31 patients (3–6, 9, 48). N283 appeared in 36% of brain-derived sequences ($n = 343$), compared with only 10% of blood and lymphoid sequences ($n = 307$; Table 2). Thus, N283 was significantly more frequent in brain than in blood or lymphoid tissues ($P < 0.01$, Fisher's exact test). Similar results were obtained when sequence data were limited to two to five sequences per patient. To determine whether N283 was associated with HAD, the dataset was expanded to include studies with brain sequences from patients with or without HAD (7, 8, 11, 12). In 481 brain sequences from 66 AIDS patients,

Table 1. Kinetic constants for gp120–sCD4 interactions

gp120	k_{on}, 10^5 $M^{-1}\cdot s^{-1}$	k_{off}, $10^{-3}\cdot s^{-1}$	K_d, 10^{-9} M
YU2	1.29 ± 0.07	0.83 ± 0.07	6.42
UK1brN	0.88 ± 0.18	2.67 ± 0.22	30.5
UK1brN N283T	1.09 ± 0.12	8.22 ± 0.67	75.6

Data were fit to a 1:1 binding model using BIAevaluation software (BIACORE).

N283 appeared in 41% of brain Envs from 41 HAD patients (n = 330) but only 8% of brain Envs (n = 151) from 25 non-HAD patients ($P < 0.001$) and was the major amino acid variant in 10 HAD patients compared with two non-HAD patients (Table 2). Furthermore, N283 was associated with compartmentalization in cerebrospinal fluid (CSF), albeit at a much lower frequency than brain, occurring in 9% of CSF sequences (n = 315) compared with 3.6% of matched blood sequences (n = 332) from 41 patients ($P < 0.01$; refs. 33–37). In other macrophage-rich tissues, N283 occurred in 22% of sequences from colon (n = 154) compared with 13% of matched lymphoid tissues (n = 154) in 14 patients (9, 38). In contrast, N283 appeared in 8% of lung-derived sequences (n = 74) and 7% of lymphoid-derived sequences (n = 83) from eight patients (9, 39). Taken together, these data suggest that N283 is associated with brain infection and HAD and may also enhance HIV replication in some macrophage-rich non-CNS tissues.

Discussion

In this study, we identified a variant in the CD4-binding site of HIV gp120, N283, that is more frequent in HIV Envs from brain (36%; n = 343) compared with lymphoid (10%; n = 307) tissues ($P < 0.01$) and is also more frequent in brain-derived Envs from HAD patients (41%; n = 330) compared with non-HAD patients (8%; n = 151; $P < 0.001$). N283 increases gp120 affinity for CD4 by decreasing the gp120-CD4 dissociation rate, enhancing the capacity of Envs to mediate fusion and entry when cells express low levels of CD4 and preferentially enhancing viral replication in macrophages and microglia. These findings suggest that Envs with N283 are associated with brain infection and dementia. This is an example of an HIV variant associated with a specific AIDS-related complication, in this case neurological disease.

Molecular modeling with the N283 variant in the JRFL crystal structure (2B4C; ref. 31) suggests that a Thr to Asn change at 283 increases the potential for a hydrogen bond to be formed with CD4 Q40. N283T viruses had a reduced ability to use low CD4 compared with parental viruses but used a Q40A mutant CD4 with similar efficiencies, suggesting that the hydrogen bond with Q40 provides an entry advantage for viruses with N283. Structural modeling suggests that Asn at 283 has more rotamers compared with Thr that may contact nearby residues in gp120 (R.L.D. and D.G., unpublished data), so N283 might also have additional effects on gp120 conformation at the Env–CD4 interface. The phenotype of Envs with N283 is context-dependent, because this variant is also present in some Envs with high CD4 dependence. BIACORE analysis showed that Envs with N283 compared with T283 have an increased affinity for CD4 due to a decreased dissociation rate from CD4. A similar mechanism was demonstrated for BORI15, a primary blood isolate adapted to replicate efficiently in microglia that has reduced dependence on CD4 (40). These findings suggest that N283 enhances HIV infection of macrophages and microglia by increasing the capacity of Envs to use the low CD4 levels expressed on these cells for fusion and entry and support a model in which HIV can acquire enhanced neurotropism by lowering the threshold levels of CD4/CCR5 required for infection. Consistent with this model, macrophage-tropic SIV variants that cause neurological disease also have an enhanced capacity to use low CD4 (41, 42). Additional determinants of neurotropism are likely to map to other Env regions (2) and may also reside in other viral genes.

Studies of single-molecule bond force spectroscopy in living cells demonstrated that gp120-CD4 binding is short-lived and weak compared with gp120-CD4 complex binding to CCR5 (43), thus allowing only a short time for the Env–CD4 complex to bind to CCR5 before dissociating. CCR5 is more mobile in the cell membrane than CD4 (44), so an increase in the amount of time for the Env–CD4 complex to colocalize with limiting amounts of CCR5 (22) would be expected to confer a highly selective advantage for entry into cells expressing low levels of both receptors by increasing the probability of a productive encounter with CCR5 and *bona fide* fusion event. Increased affinity for CD4 and CCR5 is frequently associated with increased sensitivity to neutralizing antibodies (45), so HIV variants with higher affinity for receptors may be able to replicate and persist more efficiently in the CNS, where neutralizing antibodies are present at lower concentrations than in peripheral tissues.

Macrophages play an important role in HIV pathogenesis (46). HIV-infected macrophages have been detected in brain, lung, gut-associated lymphoid tissues, spleen, and bone marrow (9, 46). Furthermore, studies using the chimeric simian-HIV model demonstrated that macrophages are the principal source of virus after $CD4^+$ T cells are depleted (47). Thus, the N283 variant, which enhances HIV replication in macrophages, may also increase viral replication in some non-CNS tissues. Our finding that N283 is present at a higher frequency in Envs from colon compared with lymphoid tissues is consistent with this prediction. The observation that N283 appeared at similar frequencies in matched lung and lymphoid tissue was unexpected, because alveolar macrophages in lung are a reservoir for HIV/SIV infection (15, 21). However, previous observations suggest that HIV infection in lung may primarily result from infection of lymphoid infiltrates (9). The finding that N283T mutations have similar effects on HIV replication in macrophages and microglia support the idea that neurotropic viruses represent a subset of macrophage-tropic HIV.

The identification and characterization of the N283 variant provide a better understanding of molecular mechanisms underlying HIV macrophage-tropism and neurotropism *in vivo* and also provide insights that may facilitate the development of therapies to prevent CNS infection and neurologic injury in HIV-infected patients. Furthermore, identification of a macrophage-tropic HIV variant that is more frequent in brain and colon compared with blood and lymphoid tissues will also help to advance future studies on the role of tissue macrophages as viral reservoirs during HIV/AIDS pathogenesis.

Materials and Methods

Virus Isolates, HIV Env Cloning, and Sequence Analysis. Primary viruses UK1-br, UK7-br, and MACS2-br were isolated from autopsy brain tissue from three AIDS patients with HAD (18). Tissues from patients UK1 and UK7 were obtained from the Edinburgh Brain Bank. Patient MACS2 was a participant in the Multicenter AIDS Cohort Study. Env genes were amplified from genomic DNA extracted from PBMC on day 7 postinfection with these primary isolates and cloned into pCR3.1 (28). Env protein expression was verified by Western blot with goat anti-gp120 (National Institutes of Health AIDS Research and Reference Reagent Program; ref. 28). Sequences were aligned by using Clustal X. N283T and T283N mutant Env plasmids and Q40A mutant pcDNA3-CD4 were created by PCR-based mutagenesis, and changes were verified by DNA sequencing.

Fusion Assays. 293T cells were cotransfected with 15 μg of Env-expressing plasmid and 2 μg of pLTR-Tat using calcium phosphate. Cf2-Luc cells (28) were cotransfected with different amounts of pcDNA3-CD4 and -CCR5 as indicated, by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The total amount of DNA in each transfection was normalized by using pcDNA3. 293T cells (2.5×10^4) and 2.5×10^5 Cf2-Luc cells were mixed in 48-well plates and incubated at 37°C for 8–12 h. Cells were lysed and assayed for luciferase activity. 293T cells cotransfected with a nonfunctional Env (pSVIII-ΔKSenv) and pLTR-Tat were used to determine background levels.

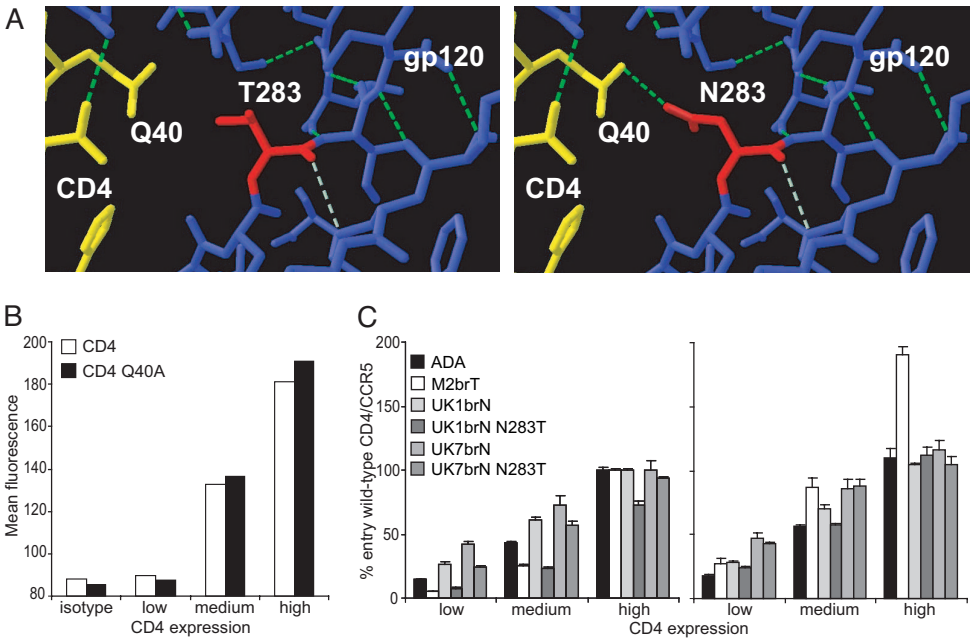

Fig. 4. Mutation Q40A in CD4 eliminates entry advantage of N283 in cells expressing low CD4. (*A*) T283N change increases potential for hydrogen bond to CD4 Q40. Swiss PDB Viewer was used to change Thr at position 283 (*Left*) to Asn (*Right*) in the JRFL gp120 crystal structure (2B4C) (31). Blue, gp120; yellow, CD4; red, position 283. Potential hydrogen bonds are indicated by dotted lines. (*B*) Cf2-Luc cells were transfected with plasmids expressing CCR5 and wild-type or Q40A mutant CD4, and CD4 cell surface expression was analyzed by flow cytometry. (*C*) Cf2-Luc cells expressing wild-type (*Left*) or Q40A mutant (*Right*) CD4 were infected with chimeric viruses expressing each Env, lysed 48 h postinfection, and analyzed for luciferase activity. Data were normalized to luciferase activity of the wild-type Env on cells expressing high wild-type CD4.

Entry Assays. The KpnI to BamHI region of *env* was PCR-amplified and cloned into pNL4–3 to generate replication-competent chimeric viruses (6). HIV luciferase reporter viruses were generated by cotransfection of 293T cells with pNL4–3env$^-$luc, an HIV provirus with *env* deleted and *nef* replaced by luciferase, and an Env-expressing plasmid, as described (28). Cf2th or Cf2-Luc cells were cotransfected with pcDNA3-CD4 and -CCR5, as above. Transfected cells were infected with 10^4 ^{3}H cpm reverse transcriptase units of virus stock. Cells were lysed 48–60 h postinfection and assayed for luciferase activity.

HIV Replication Kinetics. PBMC (3×10^6) were prepared as described (18) and incubated with 5×10^4 ^{3}H cpm reverse transcriptase units of virus stock for 3 h at 37°C. MDM were purified from PBMC by plastic adherence and cultured in RPMI medium 1640 containing 10% FBS and 10 ng/ml macrophage colony-stimulating factor, as described (18, 28), in 24-well tissue culture plates and incubated with 2×10^4 ^{3}H cpm reverse transcriptase (RT) units for 3 h at 37°C. Primary human fetal brain cultures containing a mixture of astrocytes, neurons, and microglia were prepared and maintained in 48-well tissue culture plates as described (18, 28) and incubated with equivalent amounts of virus stock (10^4 ^{3}H cpm RT units) for 16 h at 37°C. Fifty percent media changes were performed every 3–7 days for 28–35 days. Virus replication was monitored by p24 ELISA (Perkin-Elmer, Boston, MA).

Env–CD4-Binding Affinity. Plasmids expressing soluble Env proteins were constructed by introducing a frameshift by mutagenesis that resulted in a truncation at position 518. Proteins were purified from supernatants of transfected 293T cells with an F105 Ab column and were concentrated and dialyzed. Env–CD4

Table 2. N283 is associated with brain infection and HIV-associated dementia

	Amino acid frequency[†]					
Comparison	Thr[‡]	Asn	Ile	Val	Ser	Other
By tissue (31 patients)						
Brain (n = 343)	0.28 (12)	0.36* (7)[§]	0.18 (6)	0.10 (4)	0.07 (2)	0.01 (0)
Lymphoid (n = 307)	0.48 (14)	0.10* (3)	0.15 (5)	0.14 (5)	0.12 (4)	0.01 (0)
Brain, by disease[¶]						
HAD (n = 330)	0.33 (17)	0.41** (10)	0.14 (9)	0.08 (3)	0.04 (2)	0 (0)
Non-HAD (n = 151)	0.54 (16)	0.08** (2)	0.25 (5)	0.04 (1)	0.08 (1)	0.01 (0)

*, $P < 0.01$; **, $P < 0.001$. P values were calculated by using Fisher's exact test.

[†]Sequences were obtained from published studies (3–6, 9, 48) and GenBank. The frequency is calculated by dividing the number of sequences with each amino acid by the total number of sequences. The number in parentheses indicates the number of patients with the amino acid as the consensus variant.

[‡]Thr is the clade B consensus. Other indicates the combined frequency of Ala and Pro.

[§]Six of seven and two of three patients with N283 as the consensus variant in brain and lymphoid tissues, respectively, had HAD.

[¶]Sequence data compiled from 41 HAD and 25 non-HAD patients.

binding experiments were performed on a BIAcore 3000 instrument (BIACORE International, Uppsala, Sweden), as described in the Fig. 6 legend (32).

Nucleotide Accession Numbers. UK7-br (clone 34) full-length Env sequences were submitted to GenBank and assigned accession nos. DQ890515–DQ890517. UK1brN, UK1brT, and M2brT Env sequences (clones 15, 30, and 13, respectively) have been published (28). UK1 and UK7 Env sequences from autopsy brain and lymphoid tissues correspond to GenBank accession numbers DQ976408–DQ976434.

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1. Gonzalez-Scarano F, Martin-Garcia J (2005) *Nat Rev Immunol* 5:69–81.
2. Dunfee R, Thomas ER, Gorry PR, Wang J, Ancuta P, Gabuzda D (2006) *Curr HIV Res* 4:267–278.
3. Gartner S, McDonald RA, Hunter EA, Bouwman F, Liu Y, Popovic M (1997) *J Hum Virol* 1:3–18.
4. Korber BT, Kunstman KJ, Patterson BK, Furtado M, McEvilly MM, Levy R, Wolinsky SM (1994) *J Virol* 68:7467–7481.
5. Morris A, Marsden M, Halcrow K, Hughes ES, Brettle RP, Bell JE, Simmonds P (1999) *J Virol* 73:8720–8731.
6. Ohagen A, Devitt A, Kunstman KJ, Gorry PR, Rose PP, Korber B, Taylor J, Levy R, Murphy RL, Wolinsky SM, Gabuzda D (2003) *J Virol* 77:12336–12345.
7. Power C, McArthur JC, Johnson RT, Griffin DE, Glass JD, Perryman S, Chesebro B (1994) *J Virol* 68:4643–4649.
8. Shapshak P, Segal DM, Crandall KA, Fujimura RK, Zhang BT, Xin KQ, Okuda K, Petito CK, Eisdorfer C, Goodkin K (1999) *AIDS Res Hum Retroviruses* 15:811–820.
9. Wang TH, Donaldson YK, Brettle RP, Bell JE, Simmonds P (2001) *J Virol* 75:11686–11699.
10. Power C, McArthur JC, Nath A, Wehrly K, Mayne M, Nishio J, Langelier T, Johnson RT, Chesebro B (1998) *J Virol* 72:9045–9053.
11. Smit TK, Wang B, Ng T, Osborne R, Brew B, Saksena NK (2001) *Virology* 279:509–526.
12. Shah M, Smit TK, Morgello S, Tourtellotte W, Gelman B, Brew BJ, Saksena NK (2006) *AIDS Res Hum Retroviruses* 22:177–181.
13. Mankowski JL, Flaherty MT, Spelman JP, Hauer DA, Didier PJ, Amedee AM, Murphey-Corb M, Kirstein LM, Munoz A, Clements JE, Zink MC (1997) *J Virol* 71:6055–6060.
14. Sharma DP, Zink MC, Anderson M, Adams R, Clements JE, Joag SV, Narayan O (1992) *J Virol* 66:3550–3556.
15. Desrosiers RC, Hansen-Moosa A, Mori K, Bouvier DP, King NW, Daniel MD, Ringler DJ (1991) *Am J Pathol* 139:29–35.
16. Peters PJ, Bhattacharya J, Hibbitts S, Dittmar MT, Simmons G, Bell J, Simmonds P, Clapham PR (2004) *J Virol* 78:6915–6926.
17. Li S, Juarez J, Alali M, Dwyer D, Collman R, Cunningham A, Naif HM (1999) *J Virol* 73:9741–9755.
18. Gorry PR, Bristol G, Zack JA, Ritola K, Swanstrom R, Birch CJ, Bell JE, Bannert N, Crawford K, Wang H, *et al.* (2001) *J Virol* 75:10073–10089.
19. Ghorpade A, Nukuna A, Che M, Haggerty S, Persidsky Y, Carter E, Carhart L, Shafer L, Gendelman HE (1998) *J Virol* 72:3340–3350.
20. Gray L, Sterjovski J, Churchill M, Ellery P, Nasr N, Lewin SR, Crowe SM, Wesselingh SL, Cunningham AL, Gorry PR (2005) *Virology* 337:384–398.
21. Mori K, Ringler DJ, Kodama T, Desrosiers RC (1992) *J Virol* 66:2067–2075.
22. Doms RW (2000) *Virology* 276:229–237.
23. Albright AV, Shieh JT, Itoh T, Lee B, Pleasure D, O'Connor MJ, Doms RW, Gonzalez-Scarano F (1999) *J Virol* 73:205–213.
24. He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, Busciglio J, Yang X, Hofmann W, Newman W, *et al.* (1997) *Nature* 385:645–649.
25. Lewin SR, Sonza S, Irving LB, McDonald CF, Mills J, Crowe SM (1996) *AIDS Res Hum Retroviruses* 12:877–883.
26. Wang J, Crawford K, Yuan M, Wang H, Gorry PR, Gabuzda D (2002) *J Infect Dis* 185:885–897.
27. Martin J, LaBranche CC, Gonzalez-Scarano F (2001) *J Virol* 75:3568–3580.
28. Gorry PR, Taylor J, Holm GH, Mehle A, Morgan T, Cayabyab M, Farzan M, Wang H, Bell JE, Kunstman K, *et al.* (2002) *J Virol* 76:6277–6292.
29. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA (1998) *Nature* 393:648–659.
30. Yamaguchi-Kabata Y, Gojobori T (2000) *J Virol* 74:4335–4350.
31. Huang CC, Tang M, Zhang MY, Majeed S, Montabana E, Stanfield RL, Dimitrov DS, Korber B, Sodroski J, Wilson IA, *et al.* (2005) *Science* 310:1025–1028.
32. Dowd CS, Leavitt S, Babcock G, Godillot AP, Van Ryk D, Canziani GA, Sodroski J, Freire E, Chaiken IM (2002) *Biochemistry* 41:7038–7046.
33. Keys B, Karis J, Fadeel B, Valentin A, Norkrans G, Hagberg L, Chiodi F (1993) *Virology* 196:475–483.
34. Kuiken CL, Goudsmit J, Weiller GF, Armstrong JS, Hartman S, Portegies P, Dekker J, Cornelissen M (1995) *J Gen Virol* 76, 175–80.
35. Shapshak P, Nagano I, Xin K, Bradley W, McCoy CB, Sun NC, Stewart RV, Yoshioka M, Petito C, Goodkin K, *et al*. (1995) *Adv Exp Med Biol* 373, 225–238.
36. Spudich SS, Huang W, Nilsson AC, Petropoulos CJ, Liegler TJ, Whitcomb JM, Price RW (2005) *J Infect Dis* 191:890–898.
37. Strain MC, Letendre S, Pillai SK, Russell T, Ignacio CC, Gunthard HF, Good B, Smith DM, Wolinsky SM, Furtado M, *et al.* (2005) *J Virol* 79:1772–1788.
38. van der Hoek L, Sol CJ, Snijders F, Bartelsman JF, Boom R, Goudsmit J (1996) *J Gen Virol* 77:2415–2425.
39. Liu Y, Tang XP, McArthur JC, Scott J, Gartner S (2000) *J Neurovirol* (6 Suppl 1):S70–S81.
40. Martin-Garcia J, Cocklin S, Chaiken IM, Gonzalez-Scarano F (2005) *J Virol* 79:6703–6713.
41. Bannert N, Schenten D, Craig S, Sodroski J (2000) *J Virol* 74:10984–10993.
42. Mori K, Rosenzweig M, Desrosiers RC (2000) *J Virol* 74:10852–10859.
43. Chang MI, Panorchan P, Dobrowsky TM, Tseng Y, Wirtz D (2005) *J Virol* 79:14748–14755.
44. Steffens CM, Hope TJ (2004) *J Virol* 78:9573–9578.
45. Burton DR, Desrosiers RC, Doms RW, Koff WC, Kwong PD, Moore JP, Nabel GJ, Sodroski J, Wilson IA, Wyatt RT (2004) *Nat Immunol* 5:233–236.
46. Gorry PR, Churchill M, Crowe SM, Cunningham AL, Gabuzda D (2005) *Curr HIV Res* 3:53–60.
47. Igarashi T, Brown CR, Endo Y, Buckler-White A, Plishka R, Bischofberger N, Hirsch V, Martin MA (2001) *Proc Natl Acad Sci USA* 98:658–663.
48. Thomas ER, Dunfee RL, Stanton J, Bogdan D, Wang J, Kunstman K, Bell JE, Wolinsky SM, Gabuzda D (2006) *Virology*, in press.